Thc/
only

A copy of the following refrence(s) is requested :

1. ANSWER 6 OF 7   BIOSIS   COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC. on STN
ACCESSION NUMBER: 1982:300038   BIOSIS
DOCUMENT NUMBER: BA74:72518
TITLE: OZONE INDUCED FORMATION OF O O' DI
                     TYROSINE CROSS LINKS IN PROTEINS.
AUTHOR(S):   VERWEIJ H; CHRISTIANSE K; VAN STEVENINCK J
CORPORATE SOURCE: SYLVIUS LAB., DEP. MED. BIOCHEMISTRY, WASSENAARSEWEG 72,
                     2333 AL LEIDEN.
SOURCE: **BIOCHIM BIOPHYS ACTA, (1982) 701 (2), 180-184.**

2.   TITLE: CHEMICAL NATURE OF MONOGENEAN SCLERITES PART 1
                        STABILIZATION OF CLAMP **PROTEIN** BY FORMATION OF
                        DI TYROSINE.
AUTHOR(S):   RAMALINGAM K
SOURCE: PARASITOLOGY, (1973) 66 (1), 1-7.
                      CODEN: PARAAE. ISSN: 0031-1820.
TITLE: CD and proton NMR studies on the side-chain
                         conformation of tyrosine derivatives and tyrosine
                         residues in di- and tripeptides
AUTHOR(S):   Juy, Michel; Lam Thanh Hung; Fermandjian, Serge
CORPORATE SOURCE: Dep. Biol., Cent. Nucl. Stud., Gif-sur-Yvette, 91191,
                         Fr.
SOURCE: **International Journal of Peptide & Protein Research
                         (1982), 20(4), 298-307**

3.   Journal of the American Chemical Society (1985),
                        107(3), 659-66

4.   **BIOCHEMICAL JOURNAL,** (2003 Mar 1) 370 (Pt 2) 729-35.

5.   Salt-**stabilized protein** formulation

            SOURCE: **Research Disclosure (1995), 370, 56-7**

Thank you !

Jekchand Saidha
Primary Examiner
Art Unit 1652. CM1. Room No. 10D05
Mail Box 10D01
(703) 305-6595

BBA 31062

OZONE-INDUCED FORMATION OF *O,O'*-DITYROSINE CROSS-LINKS IN PROTEINS

HENDRIK VERWEIJ, KARMINAH CHRISTIANSE and JOHNNY VAN STEVENINCK

Sylvius Laboratories, Department of Medical Biochemistry, Wassenaarseweg 72, 2333 AL Leiden (The Netherlands)

Treatment of spectrin, insulin, glucagon and ribonuclease with ozone results in covalent cross-linking of these proteins. This cross-linking is not reversed by treatment with dithiothreitol and thus can not be ascribed to -S-S- bond formation. A concomitant *O,O'*-dityrosine formation is observed by spectrofluorometric analysis of the protein and by amino acid analysis and thin-layer chromatography of hydrolyzed protein samples. It is highly probable that the observed protein cross-linking should be attributed to interpeptide *O,O'*-dityrosine bonds. Several authors have shown before that oxidation of proteins with horseradish peroxidase and H_2O_2 also leads to *O,O'*-dityrosine formation. Peroxidase-induced *O,O'*-dityrosine formation in galactose oxidase (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9) causes a strong increase of enzyme activity. In accordance with these observations ozone treatment of galactose oxidase also leads to *O,O'*-dityrosine formation with a concomitant 8-fold increase of enzyme activity.

Introduction

Various toxic effects of ozone on biological systems have been described in recent literature, including deleterious effects on lung tissue [1–6], membrane damage of erythrocytes [7–10] and inhibition of several enzymes [11–14]. In attempts to explain ozone toxicity on a molecular level it has been shown that ozone may cause, among other things, lipid peroxidation [12,13,15] and covalent protein cross-linking [12,16]. It has been suggested that this ozone-induced cross-linking is caused by a reaction of proteins with lipid peroxidation aldehyde products [12], but in recent studies we have shown that this hypothesis is untenable. One of the main arguments was that ozone treatment of solubilized, lipid-free spectrin caused extensive cross-linking [16]. Further it was shown that the mechanisms of ozone-induced and photodynamic protein cross-linking are quite different [17,18].

In further experiments it was tried to elucidate the m lecular background of ozone-induced pro-

tein cross-linking. As shown in the present paper, ozone treatment of proteins leads to *O,O'*-dityrosine generation. Therefore it seems highly probable that ozone-induced protein cross-linking should be ascribed to the formation of interpeptide dityrosine bridges.

Materials and Methods

Galactose oxidase (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9) and insulin were obtained from Sigma, ribonuclease from Boehringer and glucagon from Serva. All other reagents were analytical grade and used without further purification. Ozone was generated with a Supelco Micro-Ozonizer in oxygen, at a rate of 2.5 μmol · min^{-1}. Protein and tyrosine solutions were exposed to 0.1–2.5 μmol O_3 per min by bubbling the gas into 3-ml samples.

Spectrin was isolated from human red blood cells as described before [17]. Protein concentrati ns were measured by the method of Lowry et al.

[19], with bovine serum albumin as a standard. Preparation of *O,O'*-dityrosine was performed according to the method of Malanik and Ledvina [20].

Gel electrophoresis of spectrin and ribonuclease was done on sodium dodecyl sulphate (0.2%)-polyacrylamide (5.6% w/v) gels as described by Fairbanks et al. [21]. Gel electrophoresis of insulin and glucagon was done on 7.5% acrylamide gels (with an acrylamide/bisacrylamide ratio of 15:1) in the presence of 8 M urea, as described by Swank and Munkres [22]. Densitometric scans of Coomassie blue stained gels were recorded on a Zeiss PMQII spectrophotometer with a scanning device. Protein cross-linking was estimated by the scan patterns.

Amino acid analysis was done on 22-h 6 M HCl hydrolysates, using a Beckman amino acid analyzer, equipped with a Dowex 50 column (40 × 0.4 cm). The gradient elution procedure as described by Malanik and Ledvina [23] was followed. Before hydrolysis galactose oxidase was converted to the apo-enzyme as described by Tressel and Kosman [24]. Thin-layer chromatography of hydrolysates was done on silica gel plates, according to the method of Aeschbach et al. [25], utilizing the solvent systems butanol-1/acetic acid/water (4:1:1, v/v) and propanol-1/ammonia 25% (7:3, v/v).

Fluorescence measurements were carried out on an Aminco Bowman spectrofluorometer. Galactose oxidase was assayed by measuring oxygen consumption as described by Tressel and Kosman [24].

Results

When spectrin was exposed to ozone, a progressive polymerization of the protein occurred, as shown in Fig. 1. Two polymerized bands were visible: one with a molecular weight between 400 and 500 kdaltons (apparently a spectrin dimer) and a second product with a molecular weight higher than 500 kdaltons, on top of the gel. This cross-linking could not be attributed to -S-S- bond formation, as the protein samples were treated with dithiothreitol prior to electrophoresis. A similar cross-linking was observed with insulin, glucagon and ribonuclease, but n t with galactose oxidase (n t shown).

Fig. 1. Ozone-induced cross-linking of spectrin as revealed by gel electrophoresis. 3 ml spectrin solution (1.0 mg/ml) in 10 mM phosphate buffer, pH 7.0, were treated with ozone (2.5 μmol/min), as described in the Materials and Methods. Exposure times in min as indicated.

Simultaneously the protein exhibited increasing fluorescence with an emission maximum of about 415 nm, when excited with light of 330 nm (Fig. 2). The fluorescence was not influenced by acid hydrolysis of the protein. A similar fluorescence was observed by Aeschbach et al. in proteins exposed to horseradish peroxidase and H_2O_2 [25]. These authors have shown that this fluorescence was caused by peroxidase-induced formation of *O,O'*-dityrosine in these proteins. In accordance, exposure of spectrin to peroxidase/H_2O_2, as described by Aeschbach et al., yielded a fluorescence indistinguishable from the ozone-induced fluorescence (Fig. 2). These results strongly suggest the presence of *O,O'*-dityrosine in spectrin after exposure to O_3.
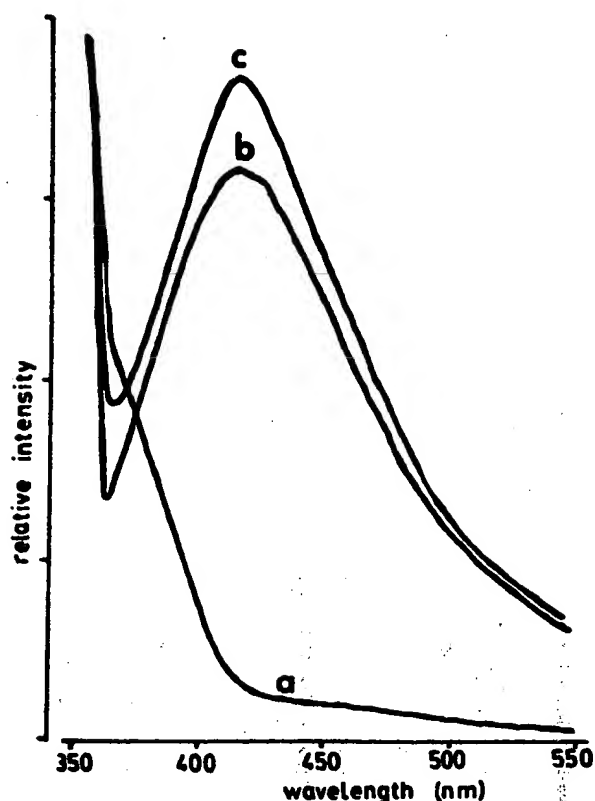
Fig. 2. Fluorescence emission spectra of (a) untreated spectrin,
(b) spectrin, treated during 5 min with ozone and (c) spectrin.
1 mg/ml 0.1 M borate buffer, pH 10, incubated during 5 min
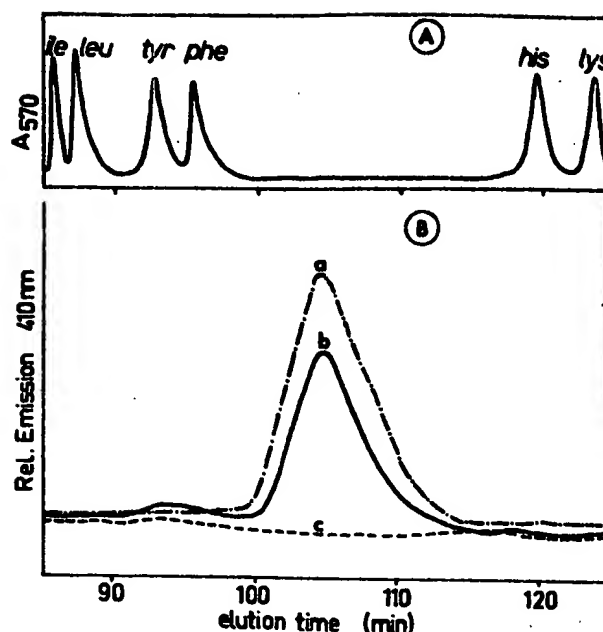with 25 mM H₂O₂ in the presence of 100 U horseradish
peroxidase/ml.

Fig. 3. Section of amino acid chromatograms of (A) standard
amino acid mixture, detection by ninhydrin absorbance and (B)
detection by fluorescence at pH 10.0 in the eluted fractions of
hydrolyzed samples of (a) O,O'-dityrosine, (b) ozone-treated
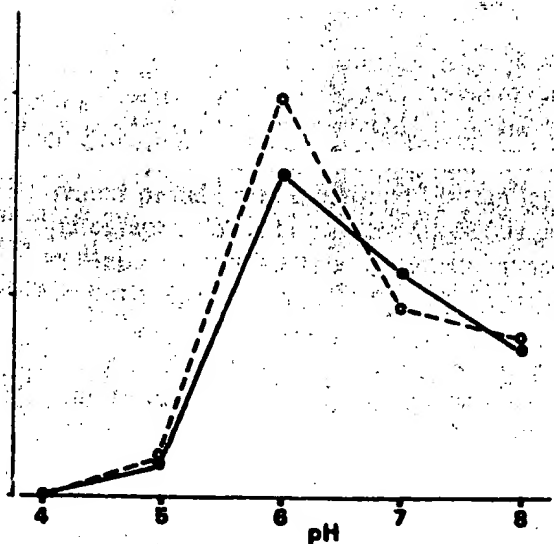spectrin and (c) untreated spectrin.

The presence of O,O'-dityrosine in ozone-
treated protein was confirmed by thin-layer chro-
matography. With both solvent systems described
in Materials and Methods a fluorescent spot, with
the same R_F value as purified O,O'-dityrosine, was
found in the hydrolysates of the oxidized proteins.

Fig. 3 shows amino acid chromatograms of
hydrolyzed spectrin and of a standard mixture.
Again O,O'-dityrosine could be identified after
exposure of the protein to ozone. Also with
ribonuclease, glucagon and insulin, O,O'-dityrosine
could only be detected after ozone exposure. With
galactose oxidase the results were slightly differ-
ent. Even the untreated protein contained a very
small amount of O,O'-dityrosine, that increased
significantly during exposure f the protein t
ozone.

Both ozone-induced cross-linking and O,O'-

Fig. 4. Ozone-induced O,O'-dityrosine formation and cross-
linking in spectrin at different pH. Conditions as in Fig. 1.
Initial velocities were calculated from the emission intensities
and from the amounts of cross-linked protein on top of the
gels, as measured by densitometric scanning. ●——●, rela-
tive velocity f O,O'-dityrosine formation: O------O, relative
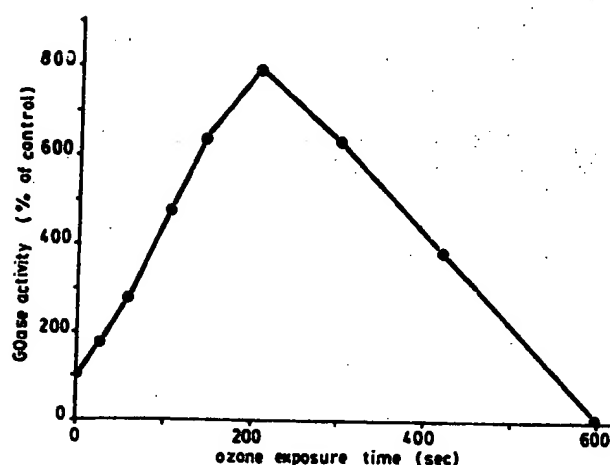velocity f cross-linking.

Fig. 5. Effect of ozone on galactose oxidase (GOase) activity, measured as described by Tressel and Kosman [24]. 2.5 ml enzyme solution (0.5 mg/ml) was treated in 10 mM phosphate buffer, pH 7.0, with 0.11 μmol ozone/min.

dityrosine formation are pH-dependent. As shown in Fig. 4, there is a close parallel between the two processes. At pH 4 no cross-linking and no O,O'-dityrosine formation were observed. At increasing pH both phenomena varied in a parallel fashion.

The influence of ozone on galactose oxidase activity is depicted in Fig. 5. With small quantities of ozone, enzyme activity was enhanced up to 800%. After longer exposure times the enzyme was inhibited.

Discussion

Cross-linking of red cell membrane proteins by ozone was interpreted by Chan et al. [12] as a reaction of these proteins with aldehyde lipid peroxidation products. In a subsequent study it was demonstrated, however, that cross-linking of spectrin also occurred in the absence of lipids, indicating that cross-linking is caused by a direct effect of ozone on the protein molecule [16].

As shown in Results, exposure of various proteins to ozone leads to O,O'-dityrosine formation. The presence of O,O'-dityrosine is ozone-treated proteins was conclusively demonstrated by the following bservations.

1. The fluorescence spectra btained with oxidized proteins (Fig. 2) were characteristic f r O,O'-dityrosine. The excitation maximum f 330

nm and the emission maximum of 410 nm were identical t those of purified O,O'-dityrosine and in accordance with previous bservations by Anderson [26].

2. Thin-layer chromatography of hydrolysates of oxidized proteins yielded a fluorescent spot with the same R_P value as purified O,O'-dityrosine.

3. Amino acid analysis on a Beckman analyzer also showed the presence of O,O'-dityrosine in oxidized proteins (Fig. 3).

O,O'-Dityrosine occurs normaly in several structural proteins [26-28]. Presumably the insolubility and elasticity of some of these proteins is, at least partially, caused by these O,O'-dityrosine cross-links. Aeschbach et al. described oxidative O,O'-dityrosine formation and concurrent cross-linking by exposure of proteins to horseradish peroxidase in the presence of H_2O_2 [25], via a reaction mechanism first described by Gross and Sizer [29]. Apparently ozone causes such a cross-linking by O,O'-dityrosine bridges in spectrin, insulin, glucagon and ribonuclease. Quantitative analysis of fluorescence of ozone-treated protein samples, utilizing O,O'-dityrosine as a standard, revealed that only a small percentage of the tyrosine residues in ozone-treated proteins was converted to O,O'-dityrosine. For instance, in the experiment shown in Fig. 1, the O,O'-dityrosine concentration was about 0.5 nmol/nmol spectrin after an exposure time of 2 min. Considering the fact that spectrin contains 44 tyrosine residues per monomer [30] and that each O,O'-dityrosine has originated from two tyrosine residues it thus appears that only 2-3% of the tyrosine residues were oxidized at this point of time. Quantitatively this O,O'-dityrosine concentration is sufficient to explain the observed cross-linking. Moreover, a strict causal relationship between ozone-induced O,O'-dityrosine formation and cross-linking is strongly suggested by the similar pH-dependences of the two phenomena (Fig. 4).

However, other possible cross-linking mechanisms are not rigidly ruled out by the described experimental results. In this context it should be emphasized that several other amino acid residues in proteins (among others, histidine and tryptophan) can be oxidized by ozone [31].

Aeschbach et al. [25] pointed out that peroxidase/H_2O_2-induced O,O'-dityrosine formation

184

leads to both inter- and intramolecular cross-links [25]. It should be expected that this also applies for ozone-induced O,O'-dityrosine formation. In this context the observations on galactose oxidase are relevant. In the untreated protein a small amount of O,O'-dityrosine was detectable, in agreement with previous observations by Tressel and Kosman [24]. These authors demonstrated that oxidation in the enzyme with peroxidase/ H_2O_2 caused a significant increase of O,O'-dityrosine content, leading to intramolecular, but not to intermolecular, cross-linking. Further it was shown that this oxidative modification resulted in stimulation of enzyme activity. There is a striking similarity between these observations and the ozone effects on galactose oxidase, as described in this paper. Ozone treatment also resulted in O,O'-dityrosine formation, without intermolecular cross-linking, with a concomitant enhancement of enzyme activity (Fig. 5).

To our knowledge enzyme activation by ozone, as demonstrated here for galactose oxidase, has not been described before for other enzymes. Most enzymes are strongly inhibited by ozone, even in very low concentrations [11–14]. It would be premature to attribute this inhibition to inter- or intramolecular O,O'-dityrosine cross-link formation, as several other amino acid residues in proteins are also susceptible to ozone-induced oxidation [31].

References

1 DeLucia, A.J., Mustafa, M.G., Hussain, Z. and Cross, C.E. (1975) J. Clin. Invest. 55, 794–802.

2 Shimasaki, H., Takatori, T., Anderson, W.R., Horten, H.L. and Privett, O.S. (1976) Biochem. Biophys. Res. Commun. 68, 1256–1262

3 Chow, C.K. (1976) Nature 260, 721–724

4 Chow, C.K., Plopper, C.G. and Dungworth, D.L. (1979) Environ. Res. 20, 309–317

5 Plopper, C.G., Dungw rth, D.L., Tyler, W.S. and Chow, C.K. (1979) Arch. Environ. Health 34, 390–395

6 Boorman, G.A., Schwartz, L.W. and Dungworth, D.L. (1980) Lab. Invest. 43, 108–115

7 Goldstein, B.D., Levine, M.R., Cuzzi-Spada, R., Cardenas, R., Buckley, R.D. and Balchum, O.J. (1972) Arch. Environ. Health 24, 243–247

8 Larkin, E.C., Kimzey, S.L. and Siler, K. (1978) J. Appl. Physiol. 45, 893–898

9 Buckley, R.D., Hackney, J.D., Clark, K. and Posin, C. (1975) Arch. Environ. Health 30, 40–43

10 Clark, K.W., Posin, C.I. and Buckley, R.D. (1978) J. Toxicol. Environ. Health 4, 741–753

11 Kindya, R.J. and Chan, P.C. (1976) Biochim. Biophys. Acta 429, 608–615

12 Chan, P.C., Kindya, R.J. and Kesner, L. (1977) J. Biol. Chem. 252, 8537–8541

13 Kesner, L., Kindya, R.J. and Chan, P.C. (1979) J. Biol. Chem. 254, 2705–2709

14 Verweij, H. and Van Steveninck, J. (1981) Biochem. Pharmacol. 30, 1033–1037

15 Freeman, B.A., Sharman, M.C. and Mudd, J.B. (1979) Arch. Biochem. Biophys. 197, 264–272

16 Verweij, H. and Van Steveninck, J. (1979) Chemosphere 7, 443–448

17 Verweij, H. and Van Steveninck, J. (1980) Biochim. Biophys. Acta 602, 591–599

18 Verweij, H., Dubbelman, T.M.A.R. and Van Steveninck, J. (1981) Biochim. Biophys. Acta 647, 87–94

19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275

20 Malanik, V. and Ledvina, M. (1979) Prep. Biochem. 9, 273–280

21 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617

22 Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462–477

23 Malanik, V. and Ledvina, M. (1979) J. Chromat. 170, 254–258

24 Tressel, P. and Kosman, D.J. (1980) Biochem. Biophys. Res. Commun. 92, 781–786

25 Aeschbach, R., Amado, R. and Neukom, H. (1976) Biochim. Biophys. Acta 439, 292–301

26 Andersen, S.O. (1963) Biochim. Biophys. Acta 69, 249–262

27 La Bella, F., Waykole, P. and Queen, G. (1968) Biochem. Biophys. Res. Commun. 30, 333–338

28 Raven, D.J., Earland, C. and Little, M. (1971) Biochim. Biophys. Acta 251, 96–99

29 Gross, A.J. and Sizer, I.W. (1959) J. Biol. Chem. 234, 1611–1614

30 Shotton, D.M., Burke, B.E. and Branton, D. (1979) J. Mol. Biol. 131, 303–329

31 Mudd, J.B., Leavitt, R., On-un, A. and McManus, T.T. (1969) Atmos. Environ. 3, 669–682